ENZYMIC TRANSFORMATIONS OF L-CYSTEINESULFINIC ACID*

by

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It was shown in the foregoing paper[2] that resting cells of *Proteus vulgaris*, strain OX-19, are capable of oxidizing L-cysteinesulfinic acid, an important intermediate product of cysteine metabolism[3], to CO_2, H_2O, and inorganic sulfate. The characteristics of the bacterial oxidation have been interpreted in terms of a dual pathway of cysteinesulfinate metabolism in the organism[2]. The bulk of evidence for this scheme has come from experiments with cell-free extracts of *P. vulgaris*, and the present paper is a detailed report of these experiments.

MATERIALS AND METHODS

P. vulgaris was grown under the conditions previously outlined[2]. Cell-free extracts were prepared by ultrasonic disintegration*** of aqueous washed cell suspensions for 10 to 13 minutes at 0°, at a frequency of 960 kilocycles and a rated power output of 11.3 watts per cm^2. Insoluble material was removed by 40 minutes centrifugation at 13,000 r.p.m. in a Servall centrifuge in the cold and the clear extract was then lyophilized. Under these conditions better than 95% cell breakage was obtained at a cell concentration of 50 to 100 mg per ml. Ultrasonic treatment in an atmosphere of inert gas failed to improve the yield of enzymes and delayed cell rupture. Extracts prepared in the Hughes press were somewhat less active, but sonic disintegration or grinding with alumina can be successfully substituted for ultrasonic treatment, as will be shown in future publications.

The compounds used in this work were obtained from the following sources: L-cysteic acid, Light Chemical Co.; brilliant cresyl blue, British Drug Houses, Ltd.; FMN† and α-ketoglutaric acid, Hoffman-LaRoche, Inc.; DPN, Schwartz Laboratories, Inc.; TPN and FAD, Sigma Chemical Co. For gifts of various materials we are greatly indebted to Dr. B. HORECKER (TPN), Dr. A. KORNBERG (nucleotide pyrophosphatase),

* Supported by a grant to this Laboratory from the Rockefeller Foundation.

** Fellows of the John Simon Guggenheim Memorial Foundation, 1951–'52. Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wisc. (U.S.A.). A preliminary account of this work has appeared[1] and a summary was presented at the Second International Congress of Biochemistry in Paris, July, 1952.

*** The instrument used was a Projecteur Ultrasonore Type L, Société de Condensation et d'Application Mécanique, Paris. We are greatly indebted to Drs. B. NISMAN AND J. SZULMAJSTER for their invaluable help in the preparation of these extracts.

† The following abbreviations are used: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; FMN, flavin mononucleotide; TRIS, tris(hydroxymethyl)aminomethane.

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and Dr. E. SORKIN (sulfinylacetic and sulfonylacetic acids). Other materials were prepared as previously described[2].

RESULTS

Oxidation of L-cysteinesulfinate in cell-free extracts. A solution of the lyophilized bacterial extract containing 6 mg protein in 3 ml 0.033 *M* TRIS buffer, pH 8.3, has a negligible endogenous respiration and, without supplementation, oxidizes cysteinesulfinate extremely slowly or not at all. Addition of 1 ml of boiled yeast extract (1 g fresh baker's yeast per ml water brought to a rapid boil and centrifuged) initiates the oxidation of the amino acid. For maximal activity, however, a suitable dye, such as brilliant cresyl blue, must be added. With excess yeast extract (1.5 ml per 3 ml reaction volume) addition of 1.5 mg of brilliant cresyl blue doubles the O_2 uptake. Other dyes tested (methylene blue, toluidine blue, FMN) enhanced the O_2 uptake, but brilliant cresyl blue was the most satisfactory catalyst and was thereafter routinely used in experiments designed to elucidate the pathway of oxidation. In its presence the O_2 consumption remained linear for at least an hour at 35° and was proportional to the protein concentration (Fig. 1) and the Q_{O_2} (40 to 45) exceeded that of the cell suspension.

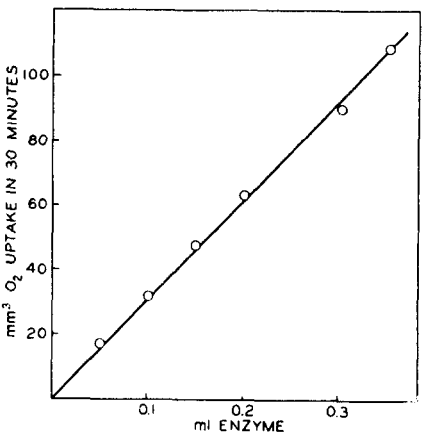

Fig. 1. Proportionality of O_2 uptake to protein concentration. Each vessel contained in a total volume of 3 ml. 100 μmoles of substrate, 1.5 mg brilliant cresyl blue, 100 μmoles TRIS buffer, pH 8.4, and 1 ml yeast extract. KOH in center cup. Temperature, 35°. Duration of experiment, 30 min. The enzyme solution used contained 16 mg protein per ml. Blank respiration without substrate has been subtracted.

Like the whole organisms, the bacterial extracts required high substrate concentration (0.03 to 0.05 *M*) and a relatively alkaline pH (8.3 to 8.6) for optimal oxidation of L-cysteinesulfinate. The enzymes responsible for the oxidation were soluble, since centrifugation for 1 hour at 18,000 r.p.m. in the high-speed attachment of the International centrifuge left the activity entirely in the supernatant solution.

Under the conditions outlined no oxidation of L-cysteate could be detected, even after supplementation with dyes or yeast extract. Thus, the enzymes responsible for its rapid oxidation in intact cells[2] were either inactivated during the ultrasonic treatment or failed to be extracted with water from the cell debris.

The effect of carbonyl fixatives. As a first effort in defining the oxidative process under study, the O_2 yield was determined in the presence of limited quantities of substrate (5 to 10 μmoles). The oxidation was necessarily a relatively slow one since the initial substrate concentration was well below the level needed for half-saturation of the enzymes. The total O_2 uptake per mole of substrate was 1.6 to 2.0 atoms (Table I); further addition of enzyme or of yeast extract failed to increase this value. The same value was obtained with and without added catalase. (The extracts were extremely rich in catalase.)

The inclusion of 0.02 *M* semicarbazide in the reaction mixture decreased the O_2

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yield per mole of substrate to 1.0 (Table I). This fact suggested that either a two step oxidation occurred, the second reaction being the oxidation of a carbonyl compound derived from cysteinesulfinate, or that more than one pathway existed for the oxidative utilization of the amino acid and one of these was blocked by the carbonyl fixative.

TABLE I

OXYGEN YIELDS OF CYSTEINESULFINATE OXIDATION BY SOLUBLE ENZYMES IN THE PRESENCE AND ABSENCE OF SEMICARBAZIDE

Substrate	*O_2 uptake on complete oxidation*	
	mm^3	*Atoms of O_2/mole CSA*
10 μM Cysteic	0	—
5 μM CSA	112.4	2.0
5 μM CSA + Semicarbazide	61.9	1.1
10 μM CSA + Semicarbazide	101.2	0.91

Experimental conditions as in Fig. 1 except that 1.7 ml yeast extract was used to insure an excess and 8 mg enzyme per vessel. Semicarbazide concentration, 0.02 *M*.

Search for the pathways of oxidation. A survey of the possible enzymic reactions into which L-cysteinesulfinate enters in the bacterial extracts revealed no measurable anaerobic or aerobic decarboxylation, anaerobic desulfination (sulfite accumulation), and only a very slight anaerobic deamination. Aerobically, significant NH_3 formation was found only in experiments of long duration; when initial reaction rates were measured, NH_3 formation accounted for only a very small fraction of the O_2 utilization. The latter observations were interpreted to mean that the primary oxidation of cysteinesulfinate is not an aerobic deamination. It may be added in this connection that our lyophilized extracts were essentially devoid of the L-amino acid oxidase discovered by STUMPF AND GREEN in a related strain of *P. vulgaris*[4].

Consideration of the structure of L-cysteinesulfinate and known facts about the metabolism of other amino acids lead to the consideration of 6 possible reactions for the initial enzymic activation of this compound.

Among the reactions listed in Table II, two are themselves dehydrogenations, but the products of all 6 might conceivably be dehydrogenated, thus accounting for the O_2 uptake found in the above experiments. Reactions 1 to 4 may be eliminated on the basis of the observations reported above. Reaction 5, the dehydrogenation of the sulfinic acid to the sulfonic acid, cannot be the *sole* reaction of cysteinesulfinate in our extracts, since the O_2 yield per mole of the latter compound is close to 2 atoms and cysteic acid is not oxidized by our extracts, while its formation entails the uptake of only 1 atom of O_2. Also, our experiments with intact cells had shown that the majority of cysteinesulfinate is not oxidized to cysteate[2]. This reasoning focussed attention on reaction 6, the transamination between cysteinesulfinate and an α-keto acid, as a possible primary reaction of cysteinesulfinate metabolism. The participation of a transaminase would also explain why NH_3 formation lagged seriously behind the O_2 uptake, although the effect of semicarbazide pointed to the formation of a carbonyl compound (β-sulfinylpyruvate) from cysteinesulfinate. Appropriate experiments were, therefore, designed to gather evidence for the possible occurrence of reactions 5 and 6.

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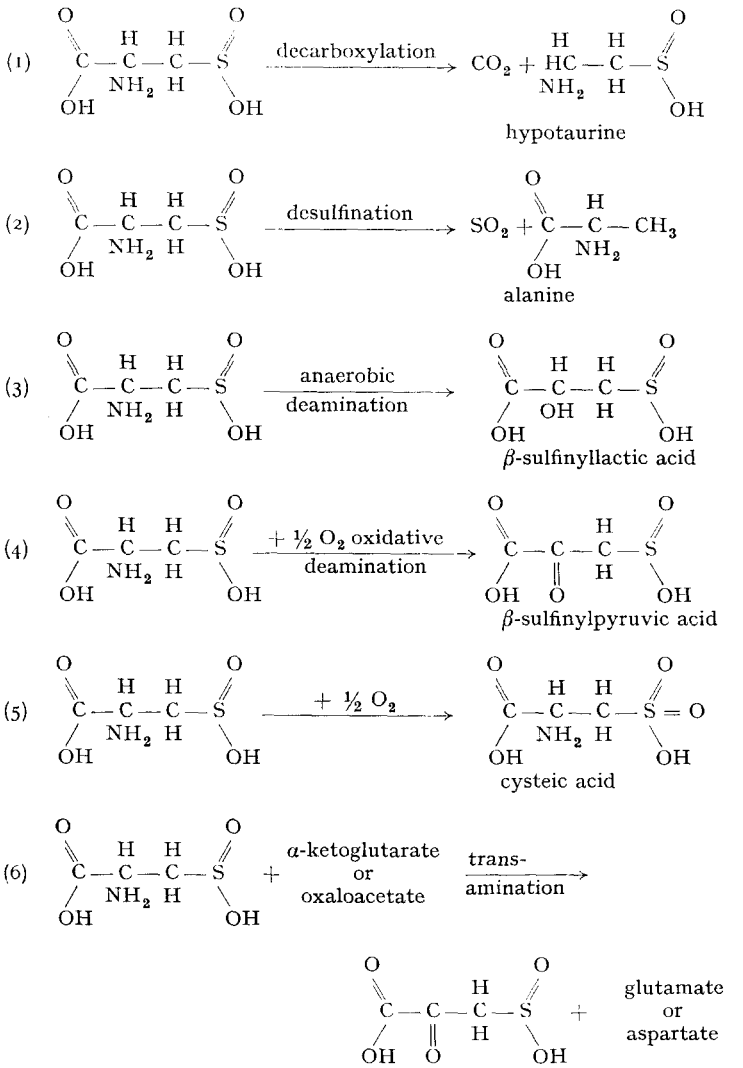
TABLE II

POSSIBLE METABOLIC REACTIONS OF CSA



Evidence for transamination. Three avenues of approach were used in the demonstration of the rôle of transaminases in the metabolism of cysteinesulfinate. First, it was considered that if β-sulfinylpyruvate is formed by transamination and is further oxidized in the system (as suggested by the effect of semicarbazide), then it should be possible to replace the yeast extract with known compounds as sources of co-factors for the oxidation. As a result of systematic elimination experiments, it was indeed established that the yeast extract supplies two substances for this pathway, α-ketoglutarate and Mn^{++}, and that at appropriate concentrations, addition of these replaces

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the yeast extract quantitatively. The original experiment establishing this fact is reproduced in Table III. It is noted that Mg^{++} fails to substitute for Mn^{++}.

TABLE III

RECONSTITUTION OF TRANSAMINATIVE PATHWAY OF CYSTEINESULFINATE OXIDATION WITH KNOWN COMPOUNDS

Additions	*mm^3 O_2 in 20 min*
Excess yeast extract	54
α-Ketoglutarate + Mn^{++}	53
No α-Ketoglutarate	0
No Mn^{++}	10
α-Ketoglutarate + Mg^{++}	10
No Cysteinesulfinate	0

Each vessel contained 5.6 mg enzyme, 70 μmoles TRIS buffer, pH 8.4, 1.5 mg brilliant cresyl blue, and 60 μmoles L-cysteinesulfinate (except the last one). α-Ketoglutarate, 40 μmoles; $MnCl_2$, 3 μmoles; $MgCl_2$, 3 μmoles, present, as noted. KOH in center cups, 35°, 3 ml total volume. The components were present at considerably suboptimal concentrations, as discussed in the text.

In this simplified assay system the O_2 utilization per mole of L-cysteinesulfinate is 1 atom, while with yeast extract a value of 1.6 to 2 atoms is reached. It seemed logical that one of the two atoms of O_2 uptake might be associated with the reoxidation of L-glutamate to α-ketoglutarate:

L-cysteinesulfinate + α-ketoglutarate $\rightleftharpoons$ β-sulfinylpyruvate + L-glutamate
L-glutamate + TPN + H_2O $\rightleftharpoons$ α-ketoglutarate + TPNH + NH_4^+.

The latter reaction would not proceed in the simplified system referred to above, for two reasons: the absence of TPN and the large amount of added α-ketoglutarate, which effectively blocks the oxidation of L-glutamate by glutamic dehydrogenase. Yeast extract, however, contains TPN and only limited quantities of α-ketoglutarate, which would permit the glutamic dehydrogenase to regenerate α-ketoglutarate, with the uptake of 1 atom of O_2.

This reasoning was verified and further evidence for the transaminative path was provided by the demonstration of the existence of a TPN-specific L-glutamic dehydrogenase in the bacterial extract (Fig. 2). The properties of the enzyme were studied by direct spectrophotometry at 340 mμ, by measuring the rate of reduction of 2,6-dichlorophenol-indophenol, with TPN as catalyst, and manometrically, in the presence of a suitable dye. It was ascertained that the activity of the enzyme is compatible with the slow uptake of the second atom of O_2 and with the slow liberation of NH_3, previously noted. As expected, the amount of α-ketoglutarate used in the simplified assay of cysteine-sulfinate oxidation by the transaminative route (Table III) completely inhibited the oxidation of L-glutamate and favoured the reduction of α-ketoglutarate to L-glutamate, in the presence of a low concentration of NH_3.

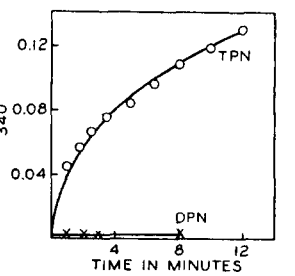

Fig. 2. Demonstration of a TPN-specific L-glutamic dehydrogenase. Ordinate, extinction at 340 mμ in 1 cm cuvette of the Beckman spectrophotometer. Abscissa, time after addition of L-glutamate. Enzyme, 0.6 mg; buffer, TRIS, pH 8.4, 50 μmole; TPN, 80 μg; DPN, 250 μg; L-glutamate, 30 μmoles; total volume, 3 ml. Temperature, 21° C.

The third line of evidence for the postulated trans-

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aminative pathway came from the direct demonstration of the accumulation of L-glutamate by means of paper chromatography. As shown in Fig. 3, in the presence of enzyme, α-ketoglutarate, and cysteinesulfinate, substantial accumulation of L-glutamate occurred. When either the amino acid or the keto acid was omitted, no glutamate was formed. Strong formation of L-glutamate was also elicited by the substitution of L-aspartate for L-cysteinesulfinate, indicating the presence of the classical α-ketoglutaric-aspartic transaminase. The previously described[5] α-ketoglutaric-cysteic acid transaminase was also present, but its activity appeared to be much weaker than that of the cysteinesulfinic transaminase. Two further points may be noted in Fig. 3. First, when the enzyme preparation was incubated with cysteinesulfinate alone, a very strong spot appeared, coinciding with cysteic acid. The enzyme responsible for the dehydrogenation of cysteinesulfinate to cysteate will be discussed in a later section and in the following paper. It may be noted, however, that addition of α-ketoglutarate always lessened the formation of cysteate, since cysteinesulfinic dehydrogenase and cysteinesulfinic-α-ketoglutaric transaminase compete for the limiting

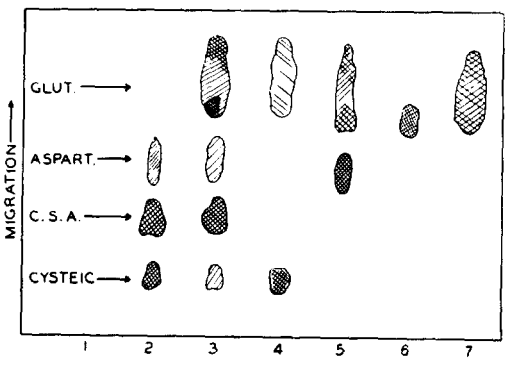

Fig. 3. Chromatography of metabolites arising from cysteinesulfinate and cysteate. 4.8 mg enzyme, 70 μM TRIS buffer, pH 8.4, and substrates, as indicated, were incubated in a volume of 3 ml for 30 min at 35°. After deproteinization with an equal volume of 10% trichloroacetic acid, 0.2 ml aliquots were concentrated and then chromatographed for 36 hours in the solvent system H_2O (1): formic acid (1.5): *n*-butanol (7.5) on Whatman No. 1 paper. Amino acids were visualized with ninhydrin. The substrates present were as follows: No. 1, α-ketoglutarate; No. 2, cysteinesulfinate; No. 3, α-ketoglutarate + cysteinesulfinate; No. 4, α-ketoglutarate + cysteate; No. 5, α-ketoglutarate + aspartate; No. 6 and No. 7, glutamate without enzyme and glutamate + trichloroacetic filtrate of enzyme. Thickness of cross-hatching indicates intensity of spot.

amount of substrate present. Second, a well-defined spot corresponding to aspartate is observable, which is lessened when α-ketoglutarate is present. This may indicate the presence of a cysteinesulfinic-oxaloacetic transaminase, oxaloacetate being provided by the slow endogenous respiration of the enzyme preparation. Whether or not this transaminase is identical with the cysteinesulfinic-α-ketoglutaric transaminase cannot be decided, but the known specificity of other transaminases and the accumulation of aspartate in the *absence* of glutamate formation strongly suggest the existence of separate enzymes. In other experiments it was ascertained that the transaminative formation of glutamate from cysteinesulfinate was uninfluenced by *added* pyridoxal phosphate.

β-Sulfinylpyruvic acid and its metabolic fate. The evidence quoted points to the transaminative formation of the keto acid corresponding to cysteinesulfinic acid. This compound, β-sulfinylpyruvic acid, to the authors' knowledge, has not been synthesized nor has it been previously implicated as a normal metabolite. As its structure indicates, the compound is an α-keto acid, analogous to oxaloacetic acid, and it may be expected to share some of the properties of the latter compound.

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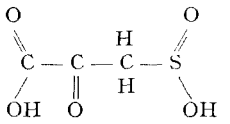

β-Sulfinylpyruvic acid

In attempting to determine the oxidative step associated with the utilization of this compound the following facts were used as a guide: 1. in the simplified system, in the presence of α-ketoglutarate and Mn^{++}, 1 atom of O_2 is taken up per mole of cysteinesulfinate; 2. anaerobically, with ferricyanide as the oxidant, in $NaHCO_3$ — H_2CO_3 buffer, 3 moles of CO_2 are released per mole of amino acid (Table IV), and the rate of CO_2 liberation from bicarbonate corresponds to the aerobic O_2 uptake; 3. of the 3 moles of CO_2, 2 are accounted for by the one atom of O_2 uptake, and the third one corresponds to the formation of a mole of acid, since no CO_2 as such is released from the substrate under either aerobic or anaerobic conditions.

TABLE IV

COMPARISON OF AEROBIC AND ANAEROBIC OXIDATIONS OF CYSTEINESULFINATE BY THE TRANSAMINATIVE PATH

Substrate	*O_2 uptake with brilliant cresyl blue, μ atoms*	*CO_2 evolution in N_2–CO_2 with ferricyanide, μmoles*	*Ratio $\frac{\mu moles\ CO_2}{\mu\ atoms\ O_2}$*
5 μM L-C.S.A.* + 30 μM α-Ketoglutarate	3.71	10.8	2.9
30 μM L-C.S.A.** + 30 μM α-Ketoglutarate	8.75	27.5	3.1

* Total manometric change after the reaction slowed to a negligible rate (180 min).

** Manometric change during first 60 min. Conditions: 12 mg enzyme, 60 μM $MnCl_2$, and substrates in 3 ml. Aerobic experiment, 0.023 M TRIS buffer; 1.5 mg brilliant cresyl blue; KOH in center cup. Anaerobic experiment, 0.067 M $NaHCO_3$; 40 μM K-ferricyanide; atmosphere, 95% N_2 — 5% CO_2. The pH was 7.8 to 7.9 in both experiments; T = 35°.

The possible metabolic reactions of β-sulfinylpyruvate, which may yield 1 atom of O_2 uptake, are listed in Table V. Of these reactions, 4. and 5. may be eliminated because of lack of CO_2 formation. Also, synthetic sulfinylacetic acid is not oxidized by *P. vulgaris* and no evidence for its formation in the extracts could be found by sensitive, chromatographic means. Reaction 1. was ruled out because of the facts that pyruvate is not oxidized by the enzyme system and aerobically no SO_2 accumulates. Reaction 3. was deemed unlikely, since β-sulfonylpyruvic acid is the end product of the metabolism of L-cysteic acid in cells of *P. vulgaris*[1,2]. It was mentioned in the previous paper[2] that the very large difference between the total O_2 uptakes on cysteinesulfinic and cysteic acids in whole cells implies that some 90% of the oxidation of the sulfinic acid proceeds by a pathway in which neither cysteic acid nor its metabolic derivatives participate. Furthermore, the formation of β-sulfonylpyruvate from β-sulfinylpyruvate would not account for the acid formation which was observed, inasmuch as pK' values of both acids are considerably lower than the pK's of H_2CO_3. This process of elimination left reaction 2 as the only likely possibility. The oxidation of $SO_3^=$ to $SO_4^=$ would explain the acid liberation observed (Table IV), since the second pK' of H_2SO_3 is higher than that of H_2CO_3, while the second pK' of H_2SO_4 is, of course, lower.

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TABLE V

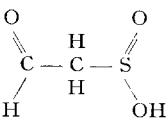
POSSIBLE METABOLIC REACTIONS OF β-SULFINYLPYRUVIC ACID

1. desulfination → SO_2 + Pyruvate → oxidation of pyruvate

2. desulfination → SO_2 + Pyruvate; oxidation of SO_2 → SO_3

3. oxidation of sulfinic group → β-Sulfonylpyruvic acid

4. oxidative decarboxylation → β-Sulfinylacetic acid + CO_2

5. anaerobic decarboxylation →  oxidation → β-Sulfinylacetate or β-Sulfonylacetaldehyde

Substantiation of this reaction scheme was accomplished as follows. First, it was shown that inorganic sulfate is formed from cysteinesulfinate under aerobic conditions and anaerobically, with ferricyanide as oxidant. Second, β-sulfinylpyruvic acid was accumulated by incubation of cysteinesulfinate and α-ketoglutarate with the enzyme at pH 8.6, in the absence of Mn^{++}. Following deproteinization (2.5 minutes heating at 90°), and subsequent addition of 10^{-3} *M* Mn^{++} in an atmosphere of N_2, the formation of $SO_3^=$ could be established by the method of FROMAGEOT *et al.*[6]. It appears, therefore, that β-sulfinylpyruvate is β-desulfinated by Mn^{++} non-enzymically, in a fashion analogous to the well-known β-decarboxylation of oxaloacetate by Mn^{++} ions.

Third, advantage was taken of the fact that Mn^{++} catalyzes the oxidation of inorganic sulfite in the absence of enzymes[7]. As shown in Fig. 4a, the Mn^{++}-catalyzed oxidation of sulfite is virtually doubled by a catalytic amount of flavin (FMN) and is inhibited by brilliant cresyl blue. The rate with FMN alone is very slow and, in the absence of both FMN and Mn^{++}, it is negligible. Other dyes, such as methylene blue, also inhibit the oxidation and the inhibitory effect is proportional to their concentration. (Possibly, this effect is exerted by reducing the effective concentration of Mn^{++}.) The addition of active enzyme fails to increase the rate of oxidation of sulfite. The catalytic effect of FMN is also shown by free riboflavin. It should be added that the enhancement of sulfite oxidation by flavins is not necessarily of physiological significance; their inclusion here serves only to accentuate the similarities between the oxidation of sulfite and of the product arising from cysteinesulfinate.

Fig. 4b depicts the characteristics of the oxidation of β-sulfinylpyruvate in our system. The keto acid was accumulated by transamination of cysteinesulfinate in the absence of added Mn^{++} and aliquots of the heat-inactivated incubation mixture were tested manometrically in the presence and absence of active enzyme. It may be noted that brilliant cresyl blue inhibits and FMN stimulates the oxidation of this compound in the same fashion as that of inorganic sulfite. The slightly greater relative rate of O_2 uptake with FMN alone may be ascribed to traces of Mn^{++} released from the proteins

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present during thermal inactivation. Addition of active enzyme failed to change the rate of O_2 uptake at optimal Mn^{++} concentration and inhibited slightly in the absence of added Mn^{++} (FMN alone), presumably owing to the ability of native proteins to complex Mn^{++} and to reduce thereby their effective concentration.

Fig. 4c demonstrates the O_2 uptake in the same system where transamination is proceeding in the course of the oxidation. Comparison with the oxidation of preformed β-sulfinylpyruvate (Fig. 4b) reveals that there is a marked lag period, which is characteristic of the transaminative formation of the β-keto acid.

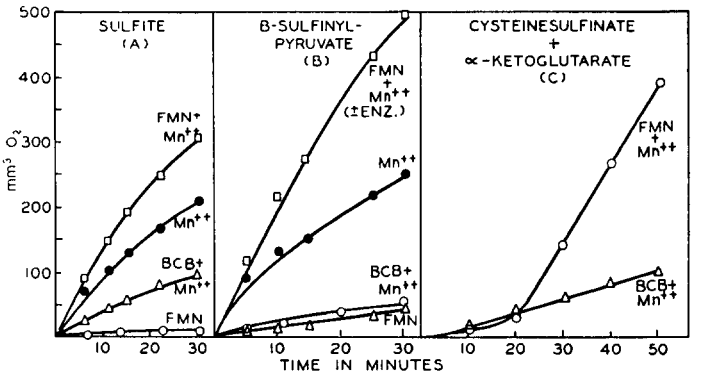

Fig. 4. Comparison of Mn^{++}-catalyzed oxidation of sulfite and of product formed by transamination. (a) Oxidation of Na_2SO_3. $SO_3^=$, 40 μM; $MnCl_2$, 6 μM; disodium riboflavin phosphate (FMN), 60 μg; brilliant cresyl blue (BCB), 1.5 mg; TRIS buffer, 170 μM; pH = 7.9; volume, 3 ml; temp., 35°. (b) Oxidation of β-sulfinylpyruvate accumulated by transamination. 720 μM DL-cysteinesulfinate + 450 μM α-ketoglutarate + 19.2 mg enzyme incubated in TRIS buffer at pH 7.9 in a total volume of 7 ml at 35° for 1 hour. The enzymes were inactivated by 2.5 minutes heating at 90°. 0.7 ml of this mixture was used as source of β-sulfinylpyruvate in exp. (b). Conditions as in exp. (a) except that the total volume was 2 ml. In one vessel 1.6 mg active enzyme was also present during assay (ENZ.). (c) Conditions as in exp. (a) except that the transamination was allowed to proceed during the manometric run. Each vessel contained $MnCl_2$ and TRIS buffer as in (a), in addition to 90 μM α-ketoglutarate and 143 μM DL-cysteinesulfinate. Brilliant cresyl blue, 1.5 mg; FMN, 600 μg.

The reasons why the sequence of events is visualized as β-sulfinylpyruvate $\xrightarrow{Mn^{++}}$ pyruvate + $SO_3^= \xrightarrow{Mn^{++}} SO_4^=$, rather than by way of reaction 3 in Table V, were given above. It should be emphasized that evidence for the intermediate formation of sulfite was obtained by the action of Mn^{++} under anaerobic conditions on accumulated β-sulfinylpyruvate. Sulfite could be shown to accumulate even aerobically, provided that brilliant cresyl blue was present to slow the rate of oxidation. Apparently, the Mn^{++} concentration optimal for sulfite oxidation is greater than that required for the desulfination step, although the latter is definitely Mn^{++}-dependent.

The experiments described provide support for reaction 2. in Table V as the correct formulation of the transaminative pathway of cysteinesulfinate oxidation in our extracts. The rôle of this series of reactions in the metabolism of intact *Proteus* cells will be considered in the DISCUSSION.

Properties of cysteinesulfinic-α-ketoglutaric transaminase. The manometric measurement of the oxidation of sulfite to sulfate served as a convenient tool for the estimation of the initial transamination between cysteinesulfinic and α-ketoglutaric acids. The presence of Mn^{++} prevents the attainment of equilibrium and the consequent slowing down of the transaminase by removing one of the products of the reaction. Advantage

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was taken of these facts in obtaining some preliminary information about the properties of the enzyme. At the time these experiments were carried out, it was not yet known that brilliant cresyl blue hinders the removal of the β-keto acid and, therefore, the dye was routinely included in the experiments.The data presented in Fig. 5 are nonetheless of value in describing the effect of experimental conditions on the transaminase.

Fig. 5a shows the sharp pH-dependence of the enzyme. At 35° optimal activity is reached at pH 9.2.That this pH effect is associated with the transamination step and not with the ensuing non-enzymic reactions is affirmed by the finding that at pH 9.2 there is no lag period in the oxidation, whereas at lower pH values (*cf.* Fig. 4c) a marked lag is noted. Figs. 5b and c show the effect of L-cysteinesulfinate and α-ketoglutarate concentrations on the rate of O_2 uptake. Half-saturation was reached at initial concentrations of $9.5 \cdot 10^{-5}$ *M* cysteinesulfinate and $5.7 \cdot 10^{-3}$ *M* α-ketoglutarate, respectively.

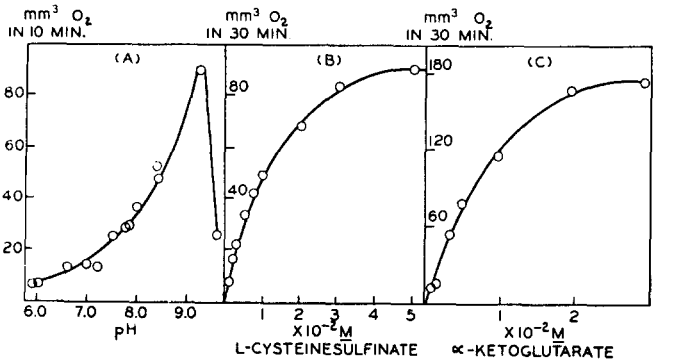

Fig. 5. Characteristics of cysteinesulfinic-α-ketoglutaric transaminase. (a) The effect of pH. Conditions as in Fig. 1, except that 90 μmoles cysteinesulfinate, 4.8 mg enzyme, 60 μmoles $MnCl_2$, and 30 μmoles α-ketoglutarate was present. Buffers, present at 0.025 *M* concentration, PO_4 (pH 5.8–7.2), TRIS (pH 7.7–8.4), NH_3 (pH 8.4–9.6). The pH values are those measured in the reaction mixture at the start of the reaction at 17°. (b) The effect of cysteinesulfinate concentration. Conditions as in (a) except the pH was 7.8 throughout. Initial substrate concentrations are recorded on the abscissa. (c) The effect of α-ketoglutarate concentration. Conditions as in (a) except that 3.2 mg enzyme was present and the pH was optimal (9.2, 0.06 *M* NH_3 buffer). Initial α-ketoglutarate concentrations are recorded on the abscissa.

A provisional value for the activity of the transaminase may be calculated from the Q_{O_2} recorded at the pH optimum of the enzyme (9.2), in the absence of brilliant cresyl blue. Since the Q_{O_2}, under these conditions, is at least 1300 and since 1 mole of cysteinesulfinate gives rise to 1 atom of O_2 consumption, the minimal value of $Q_{\text{transaminase}}$ is 2600*. This figure is considerably in excess of the values reported by COHEN *et al.*[8,9] for the activities of the L-glutamic-oxaloacetic transaminases in several species of bacteria, higher plants, and animal tissues.

Alternate pathway of cysteinesulfinate oxidation. The following circumstances indicate that oxidation of L-cysteinesulfinate occurs also by another pathway, in addition to the transaminative route: 1. with yeast extract as the source of cofactors, brilliant cresyl blue doubles the O_2 uptake, while in the reconstructed transaminase system the dye is inhibitory; 2. with yeast extract the O_2 yield per mole of cysteinesulfinate is usually less than 2 atoms (the theoretical value for the oxidation of $SO_3^=$ to $SO_4^=$ and of L-

* $Q_{\text{transaminase}} = \dfrac{\mu\text{l amino acid transaminated}}{\text{mg protein} \times \text{hr}}$

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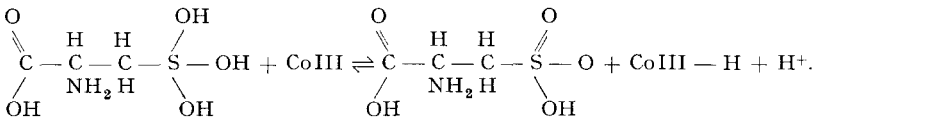
glutamate to α-ketoglutarate); 3. $2 \cdot 10^{-2}$ *M* semicarbazide, which effectively blocks the transaminative path, permits rapid oxidation of cysteinesulfinate in the presence of yeast extract, with an O_2 yield of 1 atom; 4. treatment of yeast extract with 1 *N* HCl at 100° for brief periods destroys about ½ of its activity as a source of cofactors, and the missing activity is not replaced by TPN + α-ketoglutarate; 5. incubation of the enzyme preparation with L-cysteinesulfinate (Fig. 4) results in the accumulation of cysteic acid.

Since of the possible reactions of cysteinesulfinic acid which may lead to O_2 uptake (Table II) all but transamination and cysteic acid formation have been eliminated, the latter was considered the alternative path of cysteinesulfinate metabolism in the extracts. The cysteic acid accumulation referred to above supported this hypothesis.

As shown in our preliminary communication[10] and presented in detail in the next paper[11], yeast extract supplies a new pyridine nucleotide coenzyme for this oxidation, which has been named coenzyme III. The basic dehydrogenation is visualized as follows:



The coenzyme was purified from yeast and its activity was followed manometrically, essentially under the conditions outlined in Fig. 1, except that $2 \cdot 10^{-2}$ *M* semicarbazide was included in order to eliminate the transaminative pathway. With the purified coenzyme both the dehydrogenation of L-cysteinesulfinate and the reduction of L-cysteate could be demonstrated spectrophotometrically[10,11], thus providing a firm basis for the existence of the new enzyme, L-cysteinesulfinic dehydrogenase, and for the postulated alternate pathway of cysteinesulfinate metabolism.

As mentioned above, L-cysteic acid is not further oxidized by the soluble bacterial extract, although it undergoes a slow transamination with α-ketoglutaric acid to yield β-sulfonylpyruvic acid. Whether its limited oxidation in intact cells of *Proteus*[1,2] is merely a transamination, followed by reoxidation of L-glutamate, remains to be determined.

Evidence for competition of pathways. With yeast extract as a source of all cofactors, the oxidation of L-cysteinesulfinate to cysteate (pathway A) may be studied in the presence of semicarbazide, while the transaminative route (pathway B) may be isolated by treatment of the yeast extract at pH 1 at 100° or with nucleotide pyrophosphatase (in order to destroy coenzyme III), followed by addition of sufficient TPN to saturate

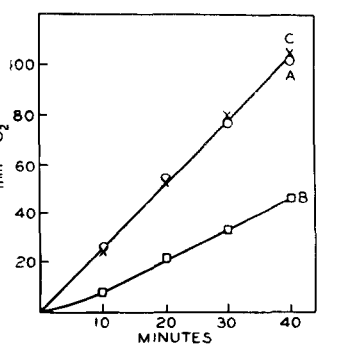


Fig. 6. Non-additiveness of pathways A and B. 4.5 ml yeast extract + 1.45 ml TRIS buffer, pH 8.4 + 0.05 ml ⬡ = 47 units highly purified nucleotide pyrophosphatase were incubated for 30 min at 35° (final pH = 7.8) and were then boiled for 2.5 minutes. Experimental conditions as in Fig. 1, except that 60 μmoles substrate, 64 μg TPN, and 1.5 ml untreated or 2.0 ml pyrophosphatase-treated yeast extract were present in a total volume of 3.5 ml. Curve A, control; Curve B, nucleotide-pyrophosphatase-treated yeast extract; Curve C, untreated yeast extract + $2 \cdot 10^{-2}$ *M* semicarbazide. 55% of the O_2 uptake in A could be ascribed to cysteinesulfinic dehydrogenase and the rest to pathway B. The latter was limited by low cysteinesulfinate concentration, unfavourable pH, and the small amounts of α-ketoglutarate + Mn^{++} present in yeast extract.

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the L-glutamic dehydrogenase. When, *in the presence of a limiting amount of* L-*cysteinesulfinate*, the rates of oxidation via the two pathways are compared with the total rate obtained without semicarbazide or nucleotide pyrophosphatase, it becomes apparent that pathways A and B are not additive (Fig. 6).

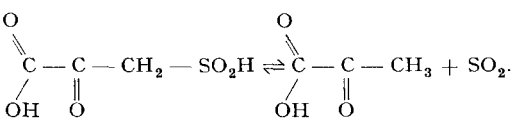
Further evidence of competition between the two pathways is seen in Fig. 4, which shows the marked suppression of cysteate formation in the presence of α-ketoglutarate. When purified coenzyme III was used in the place of yeast extract and semicarbazide was omitted, at low levels of cysteinesulfinate, the addition of large amounts of α-ketoglutaric acid (no Mn^{++}) virtually abolished the O_2 uptake.

Since both cysteinesulfinic dehydrogenase and the transaminase have high substrate saturation requirements, which are not likely to be met under physiological conditions, this competition is of significance in the oxidation of L-cysteinesulfinic acid by intact cells of the organism.

DISCUSSION

The evidence presented indicates that two new enzymes, L-cysteinesulfinic dehydrogenase and L-cysteinesulfinic-α-ketoglutaric transaminase initiate the metabolism of L-cysteinesulfinate in soluble extracts of *P. vulgaris*. The formation of aspartic acid from L-cysteinesulfinic acid points to the probable existence of a third new enzyme, L-cysteinesulfinic-oxaloacetic transaminase in the organism, but the properties of this protein have not yet been examined.

The experimental findings on the metabolism of L-cysteinesulfinic and L-cysteic acids in extracts and intact cells of the organism have been integrated into a scheme, which has already been presented[1,2]. The scheme accounts satisfactorily for all the facts known about the metabolism of these two amino acids in bacterial extracts and its applicability to the events in living cells of *Proteus* is indicated by the characteristics of the oxidation in whole cells (lag phase, Mn^{++} effect, high substrate requirements) which parallel the properties of the soluble system[2]. In the extract, the desulfination of β-sulfinylpyruvate and the oxidation of $SO_3^=$ to $SO_4^=$ are rapid, Mn^{++}-catalyzed, non-enzymatic reactions. Addition of Mn^{++} to thoroughly washed cells of *P. vulgaris* causes a dramic increase in L-cysteinesulfinate oxidation, but this does not necessarily indicate that these reactions are entirely non-enzymic in intact cells. The existence in the bacteria of Mn^{++}-requiring enzymes concerned with these two reactions cannot be ruled out. The search for an enzyme capable of desulfinating β-sulfinylpyruvic acid in bacteria and in other cells may be rewarding, in view of the probable metabolic significance of the hitherto neglected metabolic analogue of oxaloacetate, β-sulfinylpyruvic acid. If a specific desulfinase is found for this compound, it may conceivably act in a reversible fashion similarly to the Wood-Werkman reaction:



The result would be the first biological mechanism for the incorporation of inorganic sulfur into organic compounds. The reaction would be thermodynamically feasible, since transamination would provide rapid removal of the β-keto acid formed and the cysteine-

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sulfinate thus formed may be in equilibrium with other sulfur-containing amino acids. The authors hope to investigate the possible existence of this type of "SO_2 fixation".

The findings on the transaminative pathway of cysteinesulfinate metabolism have been recently extended to the metabolism of higher animals by CHATAGNER *et al.*[12]. Since a coenzyme III-dependent dehydrogenation of L-cysteinesulfinate has been found in a variety of animal tissues*, both pathways A and B may be present in higher animals, as well as in bacteria. The metabolic scheme presented[2] may thus be of wide applicability in living organisms. In this connection, tribute should be paid to PIRIE, who, on the basis of meagre data, almost 20 years ago accurately predicted that the desulfination of β-sulfinylpyruvic acid is the reaction responsible for the oxidative formation of sulfate from cysteine and cystine in animal tissues[13].

The authors are deeply indebted to Prof. C. FROMAGEOT for his hospitality and enthusiastic support of this work and for stimulating, critical discussions.

SUMMARY

1. A soluble enzyme preparation, obtained from *Proteus vulgaris* by ultrasonic disintegration, in the presence of suitable cofactors, catalyzes the rapid oxidation of L-cysteinesulfinic acid. The metabolism of the latter compound is shown to proceed by two simultaneous and competing pathways. In pathway A it is oxidized to cysteic acid and the latter is transaminated with α-ketoglutarate. Pathway B is initiated by transamination of cysteinesulfinate with α-ketoglutarate (or oxaloacetate). The resulting β-sulfinylpyruvic acid is desulfinated to pyruvate and $SO_3^=$ and the latter is oxidized to $SO_4^=$, while α-ketoglutarate is regenerated under the influence of a TPN-specific glutamic dehydrogenase.

2. Three new enzymes are described: L-cysteinesulfinic dehydrogenase (the enzyme responsible for the formation of cysteic acid), oxaloacetic-cysteinesulfinic and α-ketoglutaric-cysteinesulfinic transaminases. The properties of the latter are discussed in some detail.

3. β-Sulfinylpyruvic acid, an analogue of oxaloacetic acid, which has not hitherto been implicated in intermediary metabolism, has been accumulated by transaminase action. This compound is desulfinated to $SO_3^=$ and pyruvate, under the influence of Mn^{++}, analogously to the β-decarboxylation of oxaloacetic acid by Mn^{++}. The desulfination is a rapid, non-enzymic reaction, as is the oxidation of $SO_3^=$ to $SO_4^=$ by Mn^{++}.

4. The possibility is discussed that enzymes may exist for the desulfination reaction and that the reversal of their action (SO_2 fixation into pyruvate) may be a mechanism for the incorporation of inorganic sulfur into organic linkages.

5. L-Cysteinesulfinic dehydrogenase requires a previously unrecognized, pyridine nucleotide coenzyme.

6. The applicability of the metabolic scheme to intact bacterial cells and to animal tissues is discussed.

RÉSUMÉ

1. Une préparation enzymatique soluble, obtenue par désintégration ultrasonique de *Proteus vulgaris*, catalyse, en présence de cofacteurs convenables, l'oxydation rapide de l'acide L-cystéinesulfinique. Le métabolisme de ce dernier peut suivre deux voies simultanées et compétitives. Selon la voie A, l'acide L-cystéinesulfinique est oxydé en acide cystéique, et ce dernier est transaminé avec l'α-cétoglutarate. La voie B débute par une transamination du cystéinesulfinate avec l'α-cétoglutarate (ou l'oxalacétate). L'acide β-sulfinylpyruvique qui en résulte est désulfiné en pyruvate et $SO_3^=$, et ce dernier est oxydé en $SO_4^=$, tandis que l'α-cétoglutarate est régénéré grâce à une glutamique-déhydrogénase.

2. Trois nouveaux enzymes sont décrits: La L-cystéinesulfinique-déhydrogénase (responsable de la formation d'acide cystéique), l'oxalacétique-cystéinesulfinique et l'α-cétoglutarique-cystéinesulfinique-transaminases. Les propriétés de ce de dernier enzyme sont décrites avec quelques détails.

3. L'acide β-sulfinylpyruvique, qui correspond à l'acide oxalacétique, et qui n'a pas jusqu'à présent pris place dans le métabolisme intermédiaire, s'accumule par action de la transaminase.

* T. P. SINGER AND E. B. KEARNEY, to be published.

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Sa désulfination en $SO_3^=$ et pyruvate, sous l'action de Mn^{++}, est analogue à la β-décarboxylation de l'acide oxalacétique par Mn^{++}. Cette désulfination est une réaction rapide, non enzymatique, comme l'oxydation de $SO_3^=$ en $SO_4^=$ par Mn^{++}.

4. Il est possible qu'il existe des enzymes catalysant la désulfination et que l'inverse de cette désulfination (fixation de SO_2 sur le pyruvate) constitue un mécanisme d'incorporation du S minéral dans des composés organiques.

5. La L-cystéinesulfinique-déhydrogénase exige un coenzyme pyridine-nucléotide inconnu jusqu'ici.

6. La possibilité d'appliquer le schéma métabolique aux cellules bactériennes intactes et aux tissus animaux est discutée.

ZUSAMMENFASSUNG

1. Ein lösliches Enzympräparat, das aus *Proteus vulgaris* durch die bei Ultrabeschallung eintretende Zersetzung erhalten wurde, katalysiert in Gegenwart geeigneter Cofaktoren die schnelle Oxydation der L-Cysteinsulfinsäure. Die Umwandlung der letzteren Verbindung verläuft, wie gezeigt wird, auf zwei gleichzeitig ablaufenden und mit einander konkurrierenden Wegen. Auf Weg A wird es zu Cysteinsäure oxydiert und die letztere wird transaminiert mit α-Ketoglutarsäure. Der Reaktionsweg B beginnt mit der Transaminierung des Cysteinsulfinates mit α-Ketoglutarat (oder Oxalacetat). Die entstehende β-Sulfinylbrenztraubensäure wird desulfiniert zu brenztraubensaurem Salz und $SO_3^=$ und das letztere wird zu $SO_4^=$ oxydiert, während das α-Ketoglutarat unter der Einwirkung einer TPN-spezifischen Glutamindehydrogenase zurückgebildet wird.

2. Drei neue Enzyme werden beschrieben: L-Cysteinsulfindehydrogenase (das für die Bildung der Cysteinsäure verantwortliche Enzym), Oxalessig-Cysteinsulfin- und α-Ketoglutar-Cysteinsulfin-Transaminase. Die Eigenschaften der letzteren werden besprochen.

3. β-Sulfinylbrenztraubensäure, ein Analogon zur Oxalessigsäure, die bis jetzt noch nicht in den Zwischenstoffwechsel einbezogen war, wurde durch die Wirkung der Transaminase angehäuft. Diese Verbindung wird unter dem Einfluss von Mn^{++} desulfiniert zu $SO_3^=$ und brenztraubensaurem Salz, analog zu der β-Decarboxylierung der Oxalessigsäure mit Mn^{++}. Die Desulfinierung ist eine rasche, nicht enzymatische Reaktion, ebenso wie die Oxydation des $SO_3^=$ zu $SO_4^=$ mit Mn^{++}.

4. Die Möglichkeit wird besprochen, dass Enzyme für die Desulfinierungsreaktion bestehen könnten und dass die Umkehr ihrer Wirkung (SO_2-Einbau in brenztraubensaurem Salz) einen Mechanismus zum Einbauen anorganischen Schwefels in organische Bindungen darstellen könnte.

5. L-Cysteinsulfindehydrogenase erfordert ein vorläufig unbekanntes Pyridinnucleotidcoenzym.

6. Die Anwendbarkeit des Umwandlungsschemas auf intakte Bakterienzellen und tierische Gewebe wurde besprochen.

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Received February 5th, 1953